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The Blood–Testis Barrier in the Rat and the Physiological Compartmentation of the Seminiferous Epithelium[1]

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Previous work has indicated that the contractile "myoid cells" surrounding the seminiferous tubules serve as a barrier to the penetration of substances into the germinal epithelium. In the present study, the nature of the junctions separating myoid cells was investigated with *en bloc* uranyl acetate staining and with lanthanum nitrate, a small electron-opaque tracer useful for testing the patency of junctions. While some of the junctional complexes exhibited a continuous interspace of 200 Å, the majority displayed occluding junctions which effectively prevented passage of perfused lanthanum into the germinal epithelium. However, in 10–15% of the cross sections of tubules examined, lanthanum did penetrate the myoid cell layer through open junctions. At these sites the tracer also entered the interspaces between the Sertoli cells and the spermatogonia clearly outlining the latter. The interspaces between the spermatogonia and the Sertoli cells are continuous with those separating adjacent Sertoli cells, but the tracer was only able to penetrate for short distances further toward the tubular lumen. High magnification electron microscopy revealed that tight junctions connected adjacent Sertoli cells a short distance from the spermatogonia. Thus, while the myoid cells constitute a significant permeability barrier, the tight junctions between Sertoli cells form a second, and apparently a more effective barrier to the penetration of substances through the germinal epithelium.

These results indicate that in the seminiferous tubules two compartments are formed by the Sertoli cells and their junctional complexes: (1) a *basal* compartment between the Sertoli junctions and the basal lamina, containing the spermatogonial population and early preleptotene spermatocytes, and (2) an *adluminal* compartment above the Sertoli junctions, containing the other germ cells. The possible functional significance of the barrier at the level of the peritubular contractile cells and of the compartmentalization of the seminiferous epithelium is discussed.

Physiological studies by Setchell (1967) and Waites and Setchell (1969) have directed attention to the existence of a permeability barrier surrounding the seminiferous tubules of the mammalian testis. By cannulating the rete testis of rams these investigators were able to collect samples of testicular fluid for analysis. It was found that proteins, while abundant in blood plasma and testicular lymph, were present in very low concentrations in rete testis fluid. Significant differences in concentration were also found in amino acids and certain ions. It was concluded, therefore, that there is a blood–testis barrier located around the seminiferous tubules which is capable of excluding

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from the lumen of these tubules many substances normally present in testicular blood and lymph.

The morphological basis of the blood–testis barrier was examined with the electron microscope by Fawcett *et al.* (1970) in the guinea pig and chinchilla. Various electron-opaque particulates were injected interstitially and their localization at different time intervals was determined by examination of electron micrographs. It was found that the larger particles, carbon and thorium dioxide, were excluded from the seminiferous tubules apparently by the surrounding layer of contractile cells. Smaller tracers such as ferritin and peroxidase, though generally excluded, did penetrate into the epithelium in certain areas of the tubules. Where they did gain access to the epithelium they entered the intercellular cleft surrounding the spermatogonia but did not penetrate more deeply toward the tubular lumen. It was suggested that the primary barrier to penetration of the seminiferous tubules is the surrounding layer of contractile cells, but where this is breached, specialized cell-to-cell junctions within the epithelium constitute a secondary barrier to passage of materials into the testicular fluid.

Although there have been several studies of the peritubular contractile cells (Clermont, 1958; Lacy and Rotblat, 1960; Leeson and Leeson, 1963; Ross, 1967) these have been concerned mainly with the ultrastructural characteristics of the cells in relation to their contractile function. Ross (1967) reported that the adjoining cell membranes were usually 180–200 Å apart but there has been no detailed study of the types of cell-to-cell junction present and hence no information is available which would bear directly upon the barrier function of this layer. Similarly, certain unusual features of the junctions between Sertoli cells have been reported (Brökelmann, 1963; Flickinger and Fawcett, 1967; Nicander, 1967) but these have not been examined at high enough resolution or using *en bloc* staining procedures to permit an assessment of the exact relations between the opposed membranes.

In the present study the nature of the junctions in the seminiferous epithelium and in the peritubular contractile layer has been studied at high magnification and correlated with the permeability of these junctions to lanthanum nitrate, a very small electron-opaque tracer useful for delineating extracellular spaces and testing the patency of narrow intercellular clefts (Revel and Karnovsky, 1967). The results demonstrate that the myoid layer constitutes a significant permeability barrier but that some of its cell-to-cell junctions are not closed and therefore permit penetration of tracers into the germinal epithelium at certain sites along the length of the tubules. The specialized junctions between Sertoli cells, on the other hand, present multiple sites of focal contact of the apposed membranes. These provide a second, and apparently a more effective barrier to the passage of large molecules through the epithelium.

MATERIALS AND METHODS

Male Sprague-Dawley rats approximately 90 days of age, obtained from the Charles River Breeding Laboratories, North Wilmington, Mass., were used throughout these experiments. The testes were fixed by perfusion employing a technique similar to that described by Fawcett *et al.* (1969). The initial fixative used for perfusion was composed of 5% glutaraldehyde buffered with *s*-collidine (Bennett and Luft, 1959). Further fixation was carried out by immersion in a solution containing 2 parts of 2% osmium tetroxide and 1 part of *s*-collidine.

In order to use lanthanum as an intercellular tracer, a modification of the procedure described by Brightman and Reese (1969) was adopted. A 2% lanthanum nitrate solution was very slowly adjusted to pH 7.6–7.8 using 0.1 N NaOH. A barely visible precipitate is observed at this pH. The lanthanum hydroxide thus prepared was then added to an equal volume of *s*-collidine-buffered glutaraldehyde. The final concentration of glutaraldehyde was varied from 2.5 to 5% in different experiments. Two hundred milliliters of the fixative mixture was perfused over a period of 30 min at a pressure of 130 cm of water. This tissue was then processed in a manner similar to that employed

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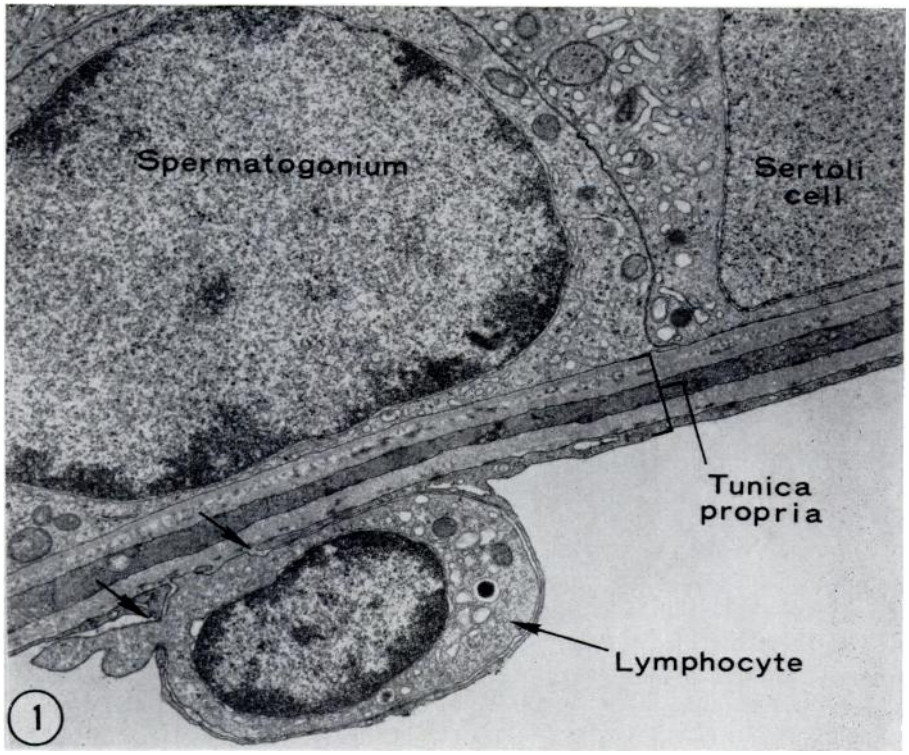

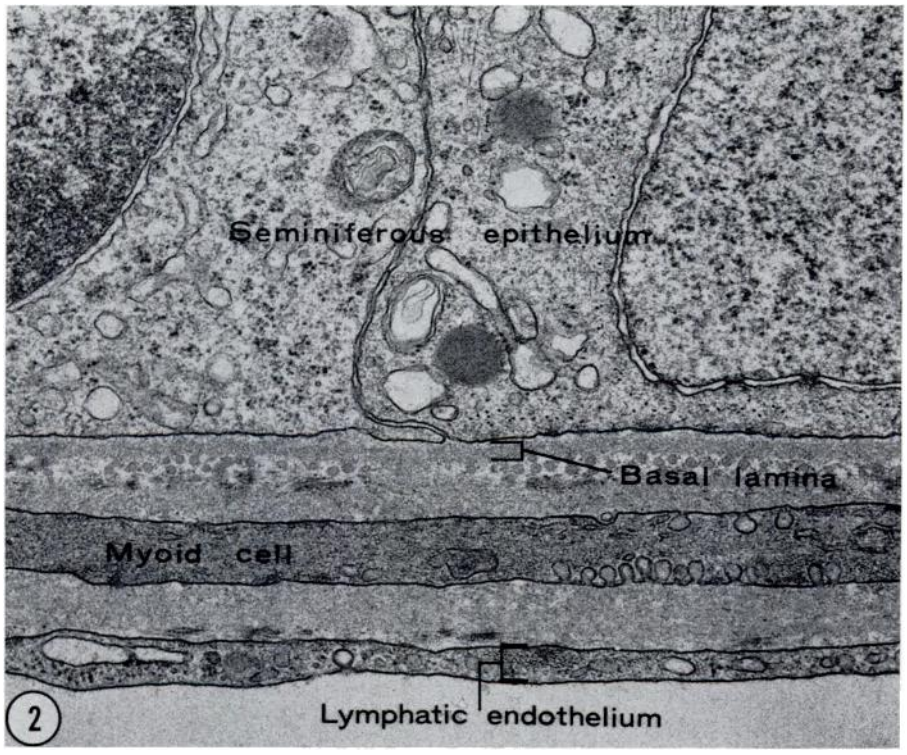

FIG. 1. Electron micrograph of a seminiferous tubule showing a spermatogonium and a Sertoli cell resting on the tunica propria. A lymphocyte has penetrated the outer lymphatic endothelium of the tunica propria (arrows). ×9500.

FIG. 2. Electron micrograph showing details of the tunica propria. The seminiferous epithelium rests on the basal lamina. Adjacent to the basal lamina collagen fibrils are seen in transverse section. The myoid cells

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for the testis not treated with lanthanum except that 2% lanthanum nitrate was now added to all solutions up to dehydration.

To enhance the contrast of membranes and to reveal their trilaminar structure to better advantage, some fixed tissue was stained *en bloc* with uranyl acetate according to the following technique (Terzakis, 1968): Subsequent to fixation, tissue blocks were washed in 0.1 M sodium acetate for 1–2 min and then immersed in 0.5% aqueous uranyl acetate (pH 3.9) for 20 min. After staining, the blocks were washed again in 0.1 M sodium acetate for 1–2 min and then dehydrated in alcohol and processed in the routine manner.

Thin sections exhibiting silver to pale gold interference colors were cut with a glass or a diamond knife for the lanthanum experiments. Thinner sections (grey to silver) were cut with a diamond knife from the tissue stained *en bloc* with uranyl acetate. The sections obtained from the lanthanum experiments were lightly stained with lead citrate (Venable and Coggeshall, 1965), while the other sections were doubly stained with uranyl acetate and lead citrate. An RCA EMU-3G or a Siemens Elmiskop I was used to examine the grids. Thick sections (0.5–1 μ) stained with toluidine blue were also prepared and the stages of the cycle of the seminiferous epithelium were classified according to the method described for the rat by Leblond and Clermont (1952).

OBSERVATIONS

The Boundary Layers of the Seminiferous Tubules. The seminiferous tubule was described by Regaud (1901) as being surrounded by a continuous layer of polygonal flattened cells. In the first electron microscopic study of the investments of the seminiferous tubules, Clermont (1958) found in cross sections a layer of contractile cells between two lamellae of carbohydrate-rich amorphous material with associated collagen fibrils. The subunits of the cellular layer were called "interlamellar cells." Peripheral to the external lamella were spindle-shaped cells which he interpreted as connective tissue cells. This general description was confirmed for the rat and mouse by several other investigators (Lacy and Rotblat, 1960; Leeson and Leeson, 1963; Ross, 1967). In a recent electron microscope study of the interstitium of the guinea pig testis, Fawcett *et al.* (1969) introduced the term "myoid cells" to emphasize the ultrastructural similarities between the peritubular contractile cells and smooth muscle cells. Their analysis of the organization of the intertubular tissue of the testis also revealed that the outer cellular layer of the boundary tissue of the tubules, which had previously been regarded as composed of fibroblasts, is in fact a layer of endothelial cells of an extensive system of peritubular lymphatic sinusoids.

The seminiferous tubules of rat testis are bounded by a constant number of clearly defined cellular and acellular layers (Figs. 1 and 2). Adjacent to the seminiferous epithelium is its basal lamina (basement membrane), a homogeneous layer of finely filamentous glycoprotein material about 0.15 μ thick. Outside of this is a clear zone containing collagen fibrils of varying orientation. Peripheral to the thin collagenous reticulum, is the inner coating of the myoid cells, a glycoprotein layer essentially identical in its fine structure to the basal lamina of the epithelium. The myoid cells meet either edge-to-edge or in overlapping junctions to form a continuous layer of epithelial-like cells. Their outer glycoprotein coating is separated by a few scattered collagen fibrils from a sheet of extremely attenuated endothelial cells that line the peritubular lymphatic sinusoids. This endothelium, like that of other lymphatics, lacks a basal lamina of its own and therefore appears to be in direct contact with the sparse collagenous reticulum associated with the outer glycoprotein investment of the myoid cells.

The myoid cells ("interlamellar cells" of Clermont, 1958; "peritubular contractile cells" of Ross, 1967) are characterized by a

(peritubular contractile cells) are characterized by numerous densely packed filaments and pinocytotic vesicles and are coated on their inner and outer surfaces by a glycoprotein material that morphologically resembles the basal lamina. Note the endothelium of a lymphatic sinusoid adjacent to the external glycoprotein coat of the myoid cell. ×27,500.

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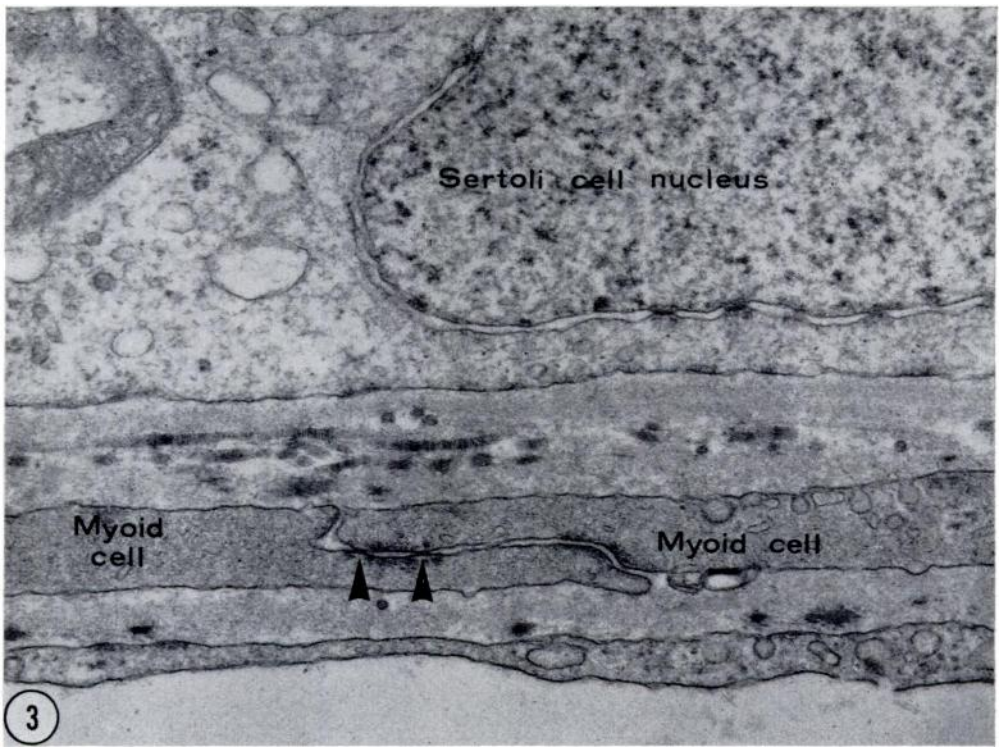

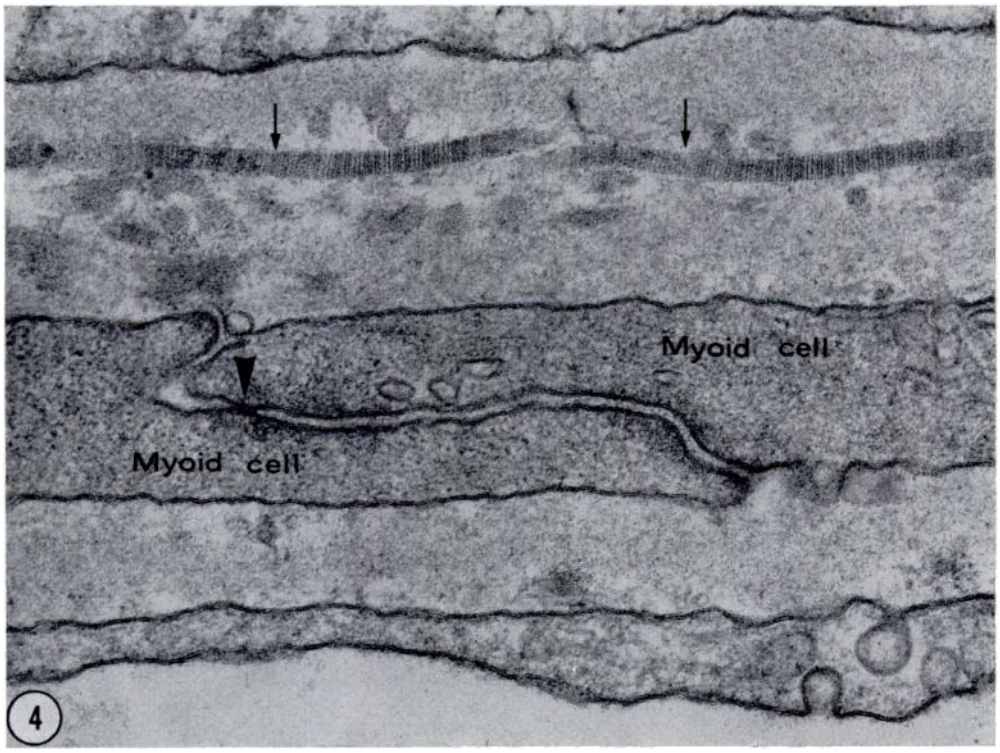

FIG. 3. Electron micrograph of adjacent myoid cells. Two tight junctions are found between the opposed cells (arrowheads). In the area of the junctional complex, the adjacent cytoplasm is more darkly stained than the rest of the cytoplasm. ×38,500.

FIG. 4. Electron micrograph of a tight junction occluding the intercellular space between two adjacent myoid cells (arrowhead). Collagen fibrils cut in longitudinal section are seen above the myoid cell layer (arrows). ×74,000. In Figs. 3 and 4 the intercellular junction is relatively long and oblique.

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cytoplasm rich in myofilaments and by numerous pinocytotic vesicles associated with the cell membranes at both the inner and outer surfaces (Fig. 2). The elliptic profile of the nucleus is centrally located in the cell, with the Golgi complex and mitochondria concentrated in the juxtanuclear cytoplasm. In the rat, as in the guinea pig (Fawcett *et al.*, 1969), there is but a single continuous sheet of myoid cells. This is in contrast to larger species such as ram, boar, and man wherein multiple layers of contractile cells appear to be the rule.

In transverse sections of seminiferous tubules the cell boundaries in the myoid layer vary in their configuration. At some sites the cell junctions extend directly across the layer and are relatively short (Figs. 5–7), while at others there is considerable overlap of the cell margins and the intercellular cleft is therefore relatively long and pursues an oblique or sinuous course (Figs. 3 and 4). In either case the apposing membranes are separated by a distance of about 200 Å throughout most of the length of the cell junction. However, in the great majority of junctions examined the intercellular cleft is narrowed at one or more sites along its length by a closer apposition of the membranes (Figs. 3–5). At these sites the adjacent cytoplasm is more darkly stained than elsewhere owing to the presence of a dense filamentous material immediately subjacent to the inner leaflet of the cell membrane.

To assess the exact relationships of the membranes requires diligent search in tissues stained *en bloc* with uranyl acetate, for areas where the layers of the opposed unit membranes are perpendicular to the plane of section and thus clearly resolved. Although these conditions are not often fulfilled, enough examples have been studied to justify the conclusion that there are limited areas of contact of the outer leaflets of the membranes (Figs. 3–5). The extracellular pathway across the myoid cell layer seems to be occluded by these tight junctions.

Occasionally junctions are encountered in which a 200-Å intercellular cleft is present across the entire thickness of the myoid cell layer (Figs. 6 and 7). Such open junctions would appear to permit free passage of materials through this cell layer. Dense filamentous material is found in the cytoplasm adjacent to such open junctions but it is not as localized as it is at sites of closer membrane apposition in the tight junctions. Extracellular material of appreciable density often occupies the intercellular cleft (Fig. 6), and in rare instances this may be concentrated in dense plaques between the outer leaflets of the cell membranes. These condensations of intercellular material may play a role in cell cohesion but there is no reason to believe that they constitute a significant obstacle to diffusion through the intercellular spaces.

Examination of the cell-to-cell junctions in the peritubular myoid layer at high magnification thus reveals that the intercellular space in the majority of junctions appears to be closed, but open junctions are occasionally found. No correlation could be determined between the stage of the cycle of the seminiferous epithelium and the type of junction found in the surrounding myoid layer.

Penetration of the Myoid Layer by Lanthanum. Following perfusion of the testis with fixative containing lanthanum nitrate, electron micrographs revealed the electron-opaque material in the interstitial capillaries and the peritubular lymphatic sinusoids. Thus, this marker readily traverses the capillary walls, permeates the extracellular spaces of the interstitium and enters the lymphatics. Penetration of the lanthanum into the seminiferous tubules is usually prevented by the myoid layer. The site of its exclusion could be identified as the points of close apposition or membrane contact between adjacent myoid

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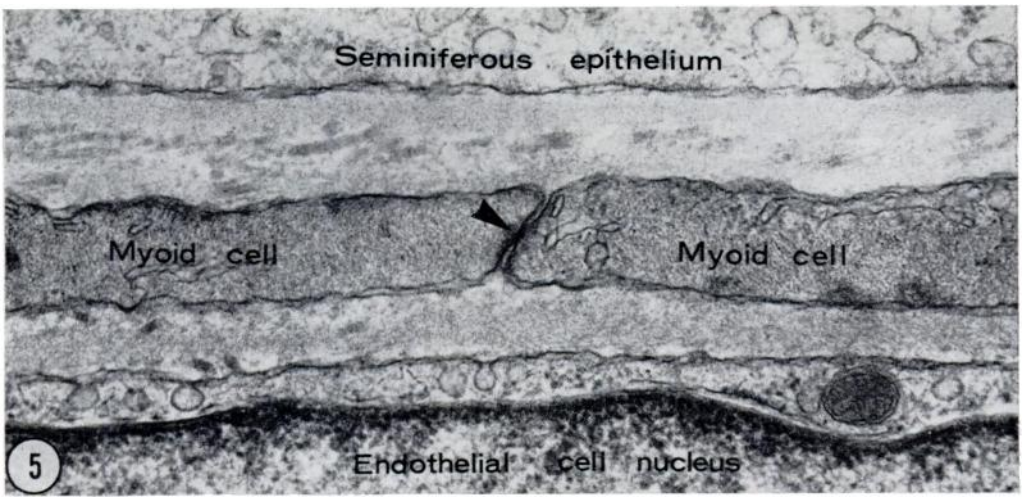

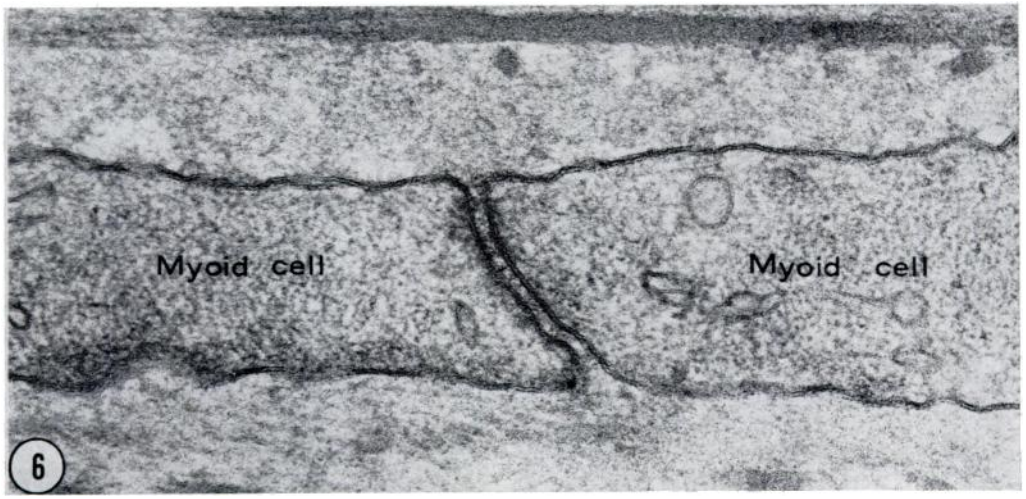

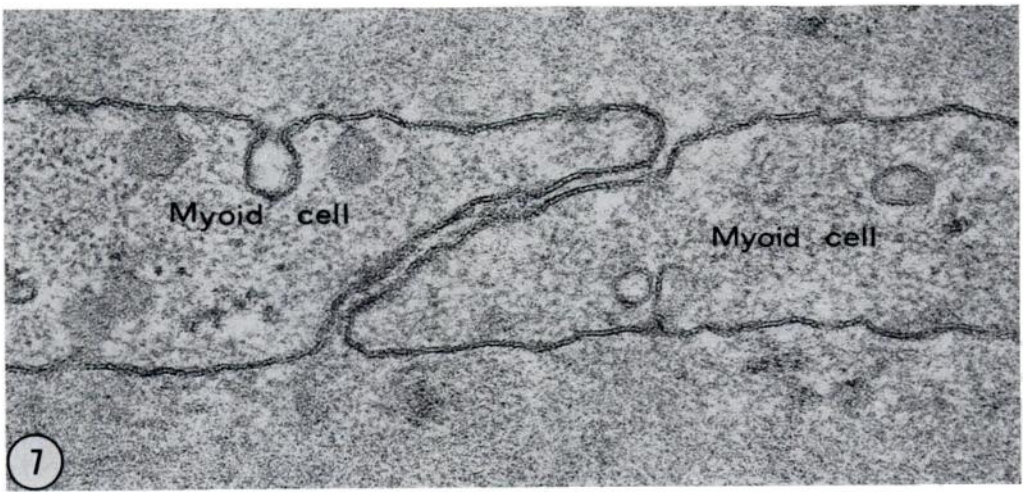

FIG. 5. Electron micrograph of a tight junction between two myoid cells (arrowhead). In contrast to the junctions in Figs. 3 and 4, this junction, as well as those depicted in Figs. 6 and 7, is almost perpendicular to the long axis of the myoid cells. The nucleus of a lymphatic endothelial cell is seen at the bottom of the micrograph. ×50,000.

FIG. 6. Electron micrograph of a junction between two myoid cells exhibiting an open intercellular space 200 Å wide. Note the dense filamentous material in the space separating the cells. ×75,000.

FIG. 7. Electron micrograph showing another 200-Å open junction between opposed myoid cells. Note the pinocytotic vesicle in the cell on the left. ×87,000.

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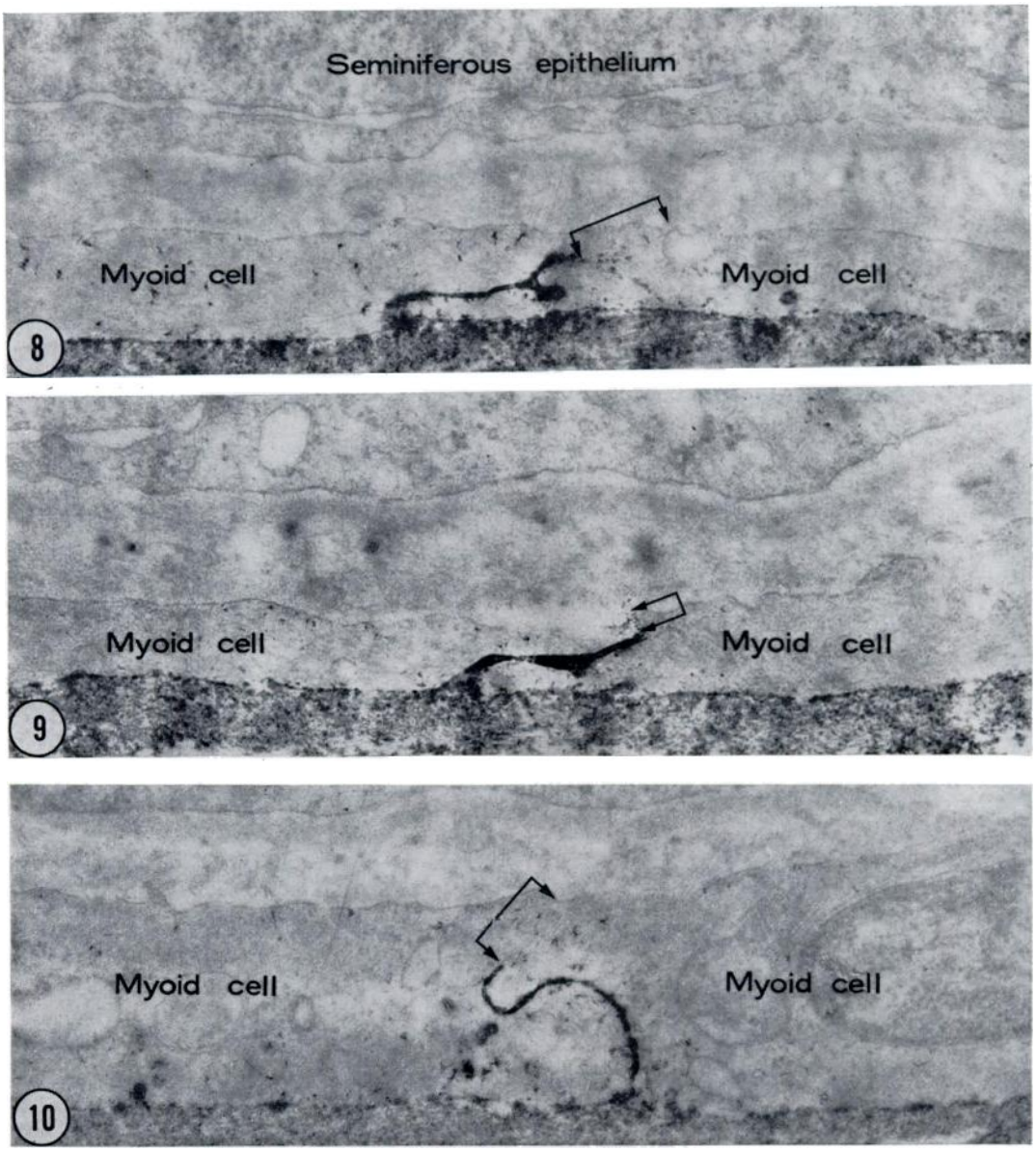

FIGS. 8–10. Lanthanum nitrate, perfused through the vascular system, readily penetrated the lymphatic sinusoids and connective tissue spaces in the intertubular area of the testis. However, the lanthanum was prevented from entering the seminiferous epithelium by junctions between the myoid cells. The lanthanum nitrate is electron-opaque and appears black in these three electron micrographs. Note that the tracer penetrates only part way through the myoid cell layer. The arrows indicate those parts of the intercellular junctions not filled with the lanthanum. Fig. 8 ×35,500; Fig. 9 ×33,000; Fig. 10 ×70,000.

cells. In favorably oriented sections the lanthanum could be seen to penetrate from the interstitium into the intercellular cleft as far as the site of close membrane apposition and there the dense column of lanthanum ended (Figs. 8–10). However, in a certain number of cross sections, estimated to represent some 10–15% of the length of

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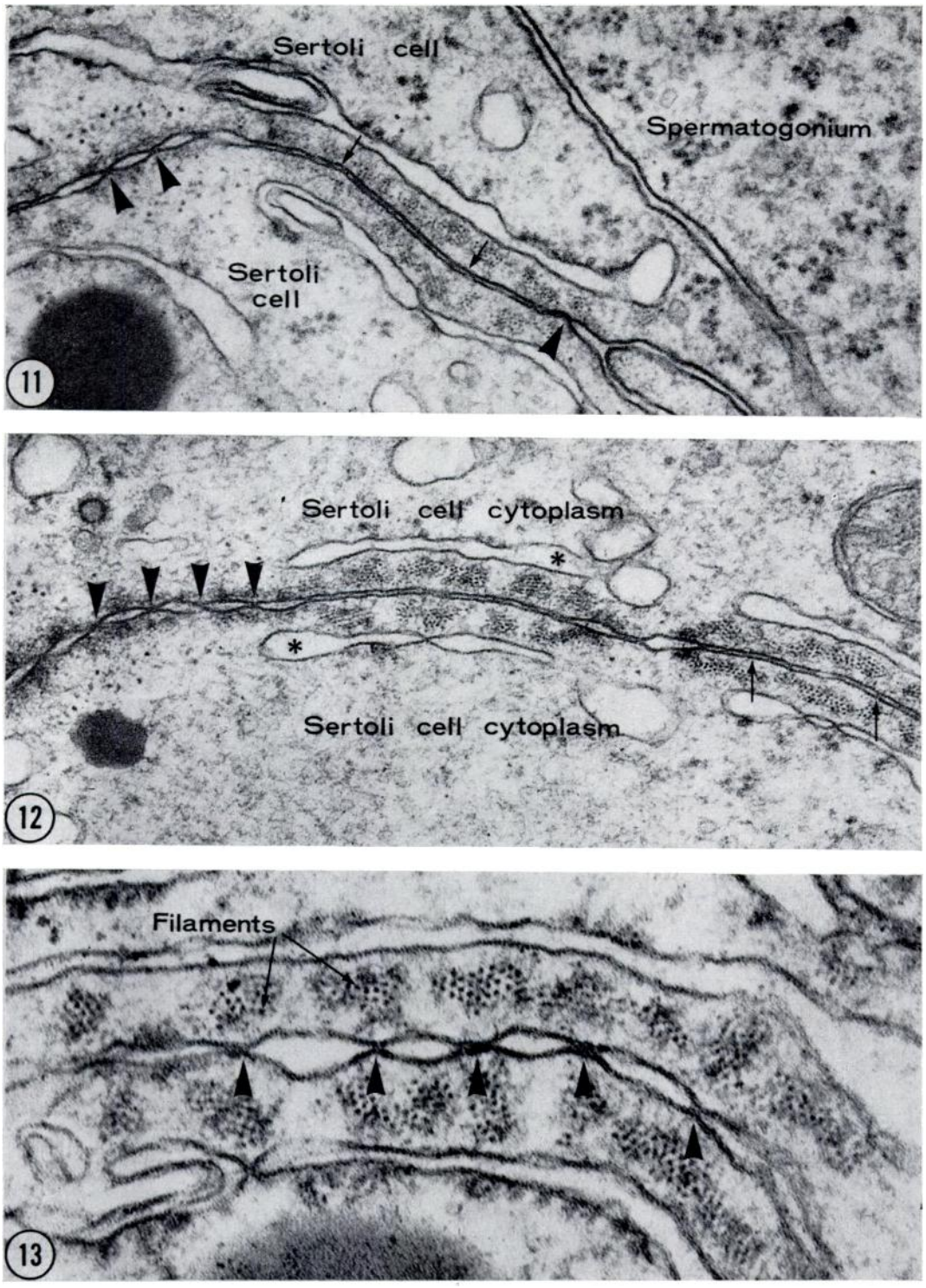

FIG. 11. Electron micrograph depicting a spermatogonium and two adjacent Sertoli cells. The tunica propria of this tubule is to the right. A simple 200-Å interspace is evident between the spermatogonium and the Sertoli cell (upper right), but the Sertoli–Sertoli interface exhibits tight junctions (arrowheads) as well as 90-Å "narrow" junctions (arrows). Bundles of filaments are seen cut in transverse section in the cytoplasm of the Sertoli cells and cisternae of endoplasmic reticulum are apparent about 500 Å from the cell surface. ×61,000.

FIG. 12. Electron micrograph of a Sertoli–Sertoli intercellular junction. Tight junctions (arrowheads) and

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the seminiferous tubules, the lanthanum did penetrate the myoid cell layer and accumulated in the space between it and the base of the germinal epithelium. It is assumed that these areas correspond to the distribution of the open junctions between myoid cells described above and illustrated in Figs. 6 and 7. It appears to us that a positive correlation exists between the fine structure of the junctional complexes separating myoid cells and our observations on the penetration by the small electron-opaque tracer, lanthanum.

The Sertoli Cells and Their Junctional Complexes. The germinal epithelium of the testis is composed of a fixed population of supporting Sertoli cells, a stem cell population of spermatogonia resting on the basal lamina, and a mobile population of developing germ cells whose topographical relations to the fixed elements are constantly changing as these cells differentiate and are displaced upward toward the lumen. As might be expected, specializations for attachment do not develop on the surfaces of contact between germ cells and supporting cells, for presumably these would interfere with the upward mobility of the germ cells. There are, however, enduring contacts and specialized junctions between adjacent members of the fixed population of supporting cells. Indeed, the formation of these junctional specializations and their extension may be a means by which the clones of germ cells are moved upward in the epithelium.

The spermatogonia are approximately hemispherical in form with their flat surfaces applied to the basal lamina of the epithelium (Fig. 1). The remainder of the base of the epithelium is made up of Sertoli cells which also rest upon the basal lamina between the spermatogonia and arch over them. In certain areas along the length of the seminiferous tubules the junctions between Sertoli cells extend to the basal lamina, but their most extensive contacts are located just above the spermatogonia (Fig. 11). It is conceptually useful to think of the spermatogonial population as being isolated from the other germ cells by the surrounding and overlying Sertoli cells in a separate compartment at the base of the epithelium (Fig. 18). As will be shown, these topographical relationships probably result in a physiologically significant isolation of the spermatogonia from the rest of the epithelium.

The spermatogonia are separated from the surrounding Sertoli cells by an intercellular cleft about 200 Å wide (Fig. 11). There are no sites of closer approximation of the cells and no desmosomes or other specializations of the opposing membranes. At one or more points on the periphery of the spermatogonium, this intercellular space is often continuous with the space separating adjoining Sertoli cells. In contrast to the uniform intercellular space surrounding the spermatogonia, the clefts between Sertoli cells vary in width and the membranes of adjoining cells exhibit diverse junctional specializations. Following such an intercellular space radially from the spermatogonium, the Sertoli cell membranes converge, narrowing the interspace to about 90 Å (Figs. 11 and 12). These areas of closer approximation are quite extensive. Immediately subjacent to the cell membrane are regularly spaced bundles of fine filaments running parallel to each other and to the cell surface. The filaments, when viewed in cross section at high magnification, usually exhibit hexagonal packing (Fig. 12). Deep to the layer of filaments in each cell and about 500 Å from the plasma membrane is a cisterna of the endoplasmic reticulum

90-Å "narrow" junctions (arrows) are apparent. Subsurface cisternae of endoplasmic reticulum (*) are separated from the cell surface by bundles of filaments that are hexagonally arranged. ×63,000.

FIG. 13. Electron micrograph of tight junctions between adjacent Sertoli cells (arrowheads). The hexagonal arrangement of the subjacent filaments is obvious. ×129,000.

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that also courses parallel to the cell surface (Figs. 11 and 12). These junctional specializations which have thus far only been found between Sertoli cells, therefore consist of two cell membranes 90 Å apart, flanked by two layers of cytoplasm containing bundles of filaments, and two subsurface cisternae of the endoplasmic reticulum. The latter may have ribosomes on their innermost membrane but not elsewhere. The continuity of the cisternae associated with these junctions varies. They are extensive in some areas but may give way to short vesicular profiles elsewhere (Flickinger and Fawcett, 1967; Nicander, 1967).

Along the course of these Sertoli–Sertoli junctions the 90-Å interspace may be further narrowed for varying distances to the 20 Å characteristic of so-called gap junctions (Figs. 14 and 15). These gap junctions can sometimes be followed in sections for distances of 7 μ or more. Filaments and subsurface cisternae are associated with these just as they are with the segments of the junction that exhibit a 90-Å interspace. The membranes are presumed to exhibit the hexagonal pattern characteristic of gap junctions in other tissues (Revel and Karnovsky, 1967) but such a pattern was not demonstrated in this study.

In addition to these junctional specializations numerous focal tight junctions are found (Figs. 11–13). At these the outer leaflets of the opposing Sertoli cell membranes come into contact, entirely obliterating the intercellular space. It is not evident whether these are punctate or whether they represent linear bands of contact that extend for some distance perpendicular to the plane of the section. In following intercellular clefts radially from the space around a spermatogonium one frequently encounters such a tight junction between the Sertoli cells a short distance from the spermatogonium (Fig. 11). This is usually followed by an extensive junctional complex with associated subsurface filaments and cisternae and 90- or 20-Å interspaces. This specialization may then be interrupted by a region with multiple focal tight junctions. Interspersed among these three types of junctions are unspecialized segments in which the Sertoli cell surfaces are separated by the usual 200-Å interspace.

The interface between Sertoli cells thus presents a great diversity of membrane relationships and associated cytoplasmic specializations. In other organs where the permeability of such junctions has been tested, the 20-Å gap junctions constitute a barrier to molecules and particulate markers larger than lanthanum, and the tight junctions completely bar access of markers to the intercellular space beyond. It is to be expected therefore that substances might penetrate the cleft around spermatogonia in the base of the seminiferous epithelium, but the extensive areas of close apposition between Sertoli cells and the focal tight junctions might well prevent deeper penetration of the epithelium. This expectation was borne out in the present study on the rat using lanthanum as an electron-dense intercellular tracer.

Penetration of Lanthanum into the Seminiferous Epithelium. It was noted above that in experiments in which the testis was perfused with fixative containing lanthanum, the electron-opaque material penetrated through open junctions in the myoid layer in some areas along the length of the seminiferous tubules. In such sites the lanthanum also penetrated into the 200-Å space between the spermatogonia and the surrounding Sertoli cells. The spermatogonia in sections were clearly outlined by a dark line resulting from the filling of this intercellular cleft by lanthanum (Figs. 16 and 17). The marker sometimes extended several micra radially into the intercellular cleft between Sertoli cells but there it was stopped abruptly, apparently by tight junctions between the Sertoli cells (Figs. 16 and 17). From the study of thin sections alone it was impossible

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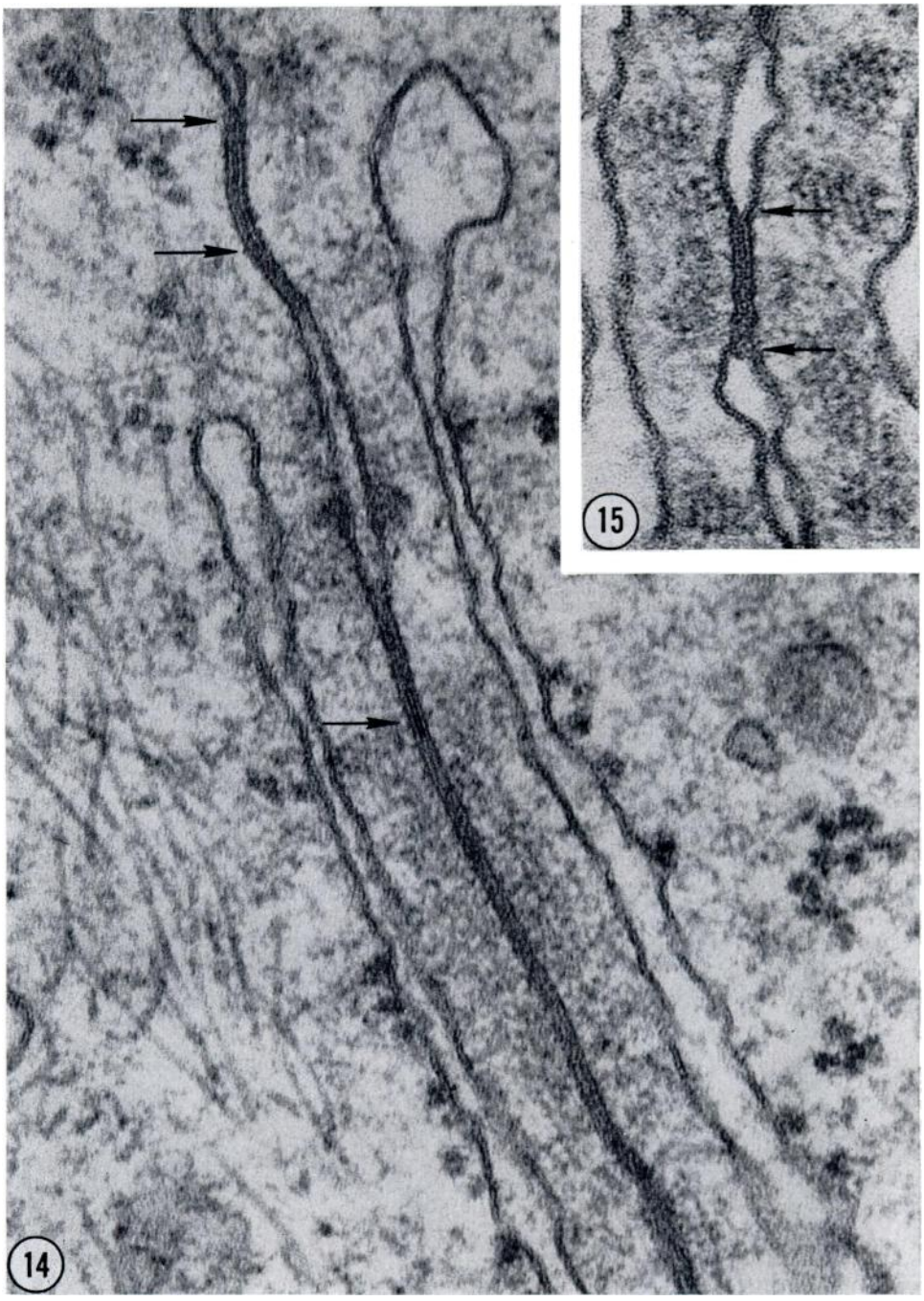

FIG. 14. Electron micrograph of a 20-Å gap junction separating adjacent Sertoli cells (arrows). There are filaments and cisternae of endoplasmic reticulum in the subjacent cytoplasm. Note the profiles of microtubules cut in longitudinal section in the lower left half of the micrograph. ×130,000.

FIG. 15. Electron micrograph showing another 20-Å gap junction (arrows) between opposed Sertoli cells. ×180,000.

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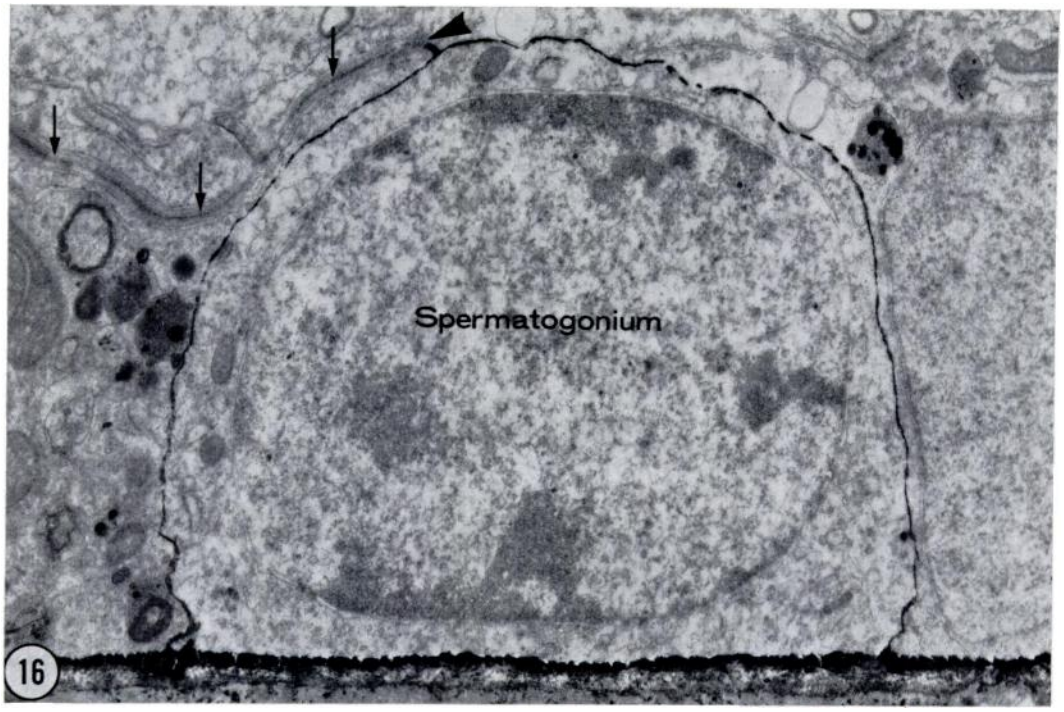

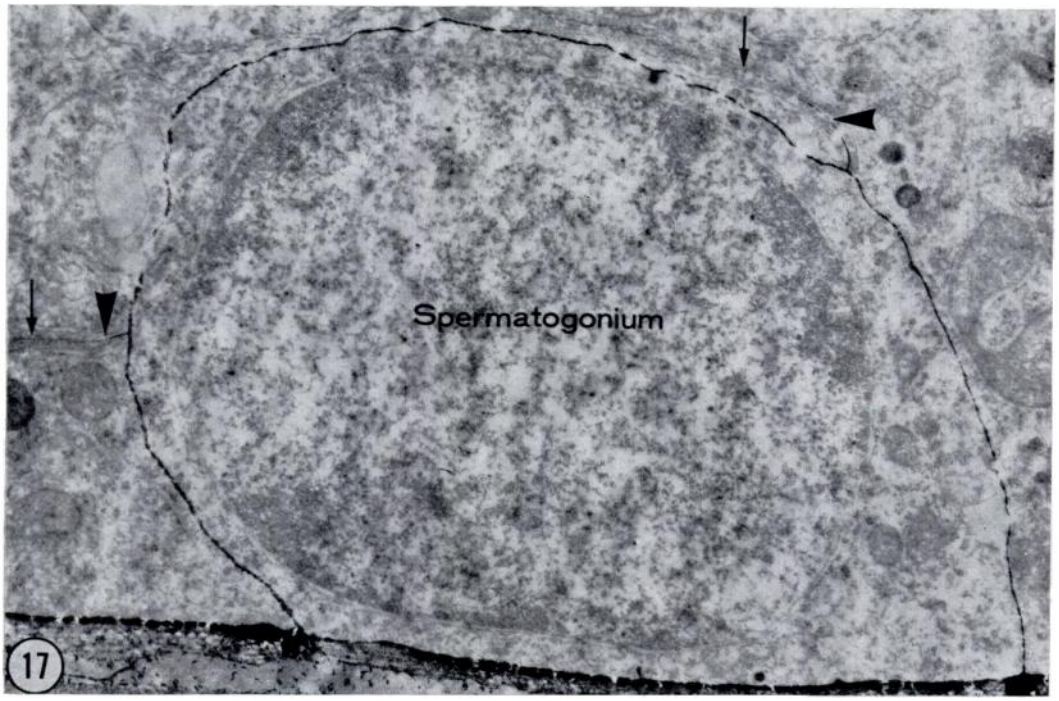

FIGS. 16 AND 17. In certain sites along the length of the seminiferous tubules the lanthanum, perfused through the blood stream, was found deep to the myoid cells. The electron-opaque tracer penetrated the open junctions in the myoid cell layer and then permeated the intercellular cleft surrounding the spermatogonia, but was prevented from further passage toward the tubular lumen by the complex junctions between Sertoli cells. These electron micrographs show the lanthanum clearly outlining the spermatogonia. Note that the tracer entered the space between two adjacent Sertoli cells (arrowheads) but that it did not permeate this cleft for more than 1 μ. Those parts of the Sertoli–Sertoli junctions to the left of the arrowheads are not filled with the lanthanum (arrows). Fig. 16 ×9750; Fig. 17 ×10,250.

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to determine whether the focal tight junctions were punctate or bands of membrane contact running for some distance along the cellular interface perpendicular to the plane of section. Failure of the lanthanum to penetrate more deeply into the germinal epithelium indicates that these occluding junctions are not easily circumvented and are more extensive than their cross-sectional appearance would suggest.

Sections of tubules representing all 14 stages of the cycle of the seminiferous epithelium have been identified and examined in an effort to determine whether permeability to lanthanum is correlated with any particular stage of the cycle. No such correlation has been found. Observations to date indicate that the sites where the myoid layer is permeable to tracers are randomly distributed.

The results of these experiments reinforce the concept of a separate compartment in the base of the germinal epithelium containing the spermatogonial population. Furthermore, the cells in this compartment appear to be more accessible to substances diffusing from the interstitium than are the other germinal elements.

DISCUSSION

The discovery of a blood–testis barrier invites comparison with the blood–brain barrier which has been more thoroughly analyzed. Microscopists studying the concentration of astrocytic foot processes immediately around the cerebral capillaries had concluded that the barrier probably resided in the capillary wall or in this perivascular cuff of glial cell processes (Gray, 1961). When the problem was investigated at higher resolution and with the aid of electron-opaque tracers (Reese and Karnovsky, 1967), it was found that the vascular endothelium of the brain was much less permeable to peroxidase than vascular endothelium elsewhere in the body, and this difference in permeability could be explained on the basis of structural differences in the junctions between endothelial cells. In cerebral capillaries, tight junctions with local contact of the outer leaflets of the apposed endothelial cell membranes were often observed, whereas in cardiac capillaries comparable junctions were less extensive and appeared to represent only sites of closer approximation rather than contact. The clefts between the astrocytic end feet were open to the continuous system of intercellular spaces surrounding the axons. It was concluded therefore, that the principal "barrier" to the passage of large molecules into the brain is at the level of the endothelial junctions in the capillary wall.

In the testis, measurements of rate of escape of labeled albumin from the blood (Everett and Simmons, 1958) and comparisons of the composition of venous blood and lymph (Lindner, 1963) provide abundant evidence for a high degree of permeability of the interstitial capillaries and venules. Electron microscopic examination of these vessels offers no evidence of extensive tight junctions that would greatly retard or prevent escape of large molecules from the blood stream (Fawcett *et al.*, 1970). Thus, the blood–testis barrier does not appear to be located in the walls of the vascular system. It has been suggested by several investigators, that the boundary tissue of the seminiferous tubules, and especially the peritubular contractile cell layer, might constitute a permeability barrier excluding certain classes of substances from the seminiferous epithelium (Rolshoven, 1936; Lacy and Rotblat, 1960; Waites and Setchell, 1969). Until very recently, however, visual demonstration of the site of the barrier has been lacking. In an electron microscopic study of the interstitium of the guinea pig testis (Fawcett *et al.*, 1969) it was noted that, in material fixed by perfusion, a uniform flocculent precipitate of protein fills the intertubular lymphatics and extends throughout the extravascular interstitial space, but is not found in the narrow

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peritubular space between the myoid cells and the epithelium of the seminiferous tubules. This finding pointed to the contractile cell layer as the probable site of the barrier to large molecules. Pursuing this further by interstitial injection of suspensions of the electron-opaque particulates, carbon and thorium dioxide, it was found that in the guinea pig these were indeed unable to traverse the myoid layer and therefore were excluded from the germinal epithelium (Fawcett *et al.*, 1970). Smaller tracers, such as ferritin and peroxidase, were not completely excluded but reached and entered the epithelium at certain sites.

In the present study we have achieved a more precise localization of the blood–testis barrier in the rat, and a more satisfying demonstration of its morphological basis by employing an improved method for preservation and staining of membranes and by using lanthanum, an electron-opaque substance of small molecular size, useful for delineating extracellular spaces. It has been shown that lanthanum permeates minute interstices but does not penetrate intercellular spaces beyond tight junctions (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Goodenough and Revel, 1970). In the testis, lanthanum introduced intravascularly moved freely into the interstitial tissue but was largely prevented from reaching the germinal epithelium by intercellular tight junctions between the myoid cells. These findings in the rat are consistent with those previously reported for the guinea pig and support the conclusion that the myoid layer is an important component of the blood–testis barrier. It is clear, however, that since open intercellular junctions were found occasionally and lanthanum did penetrate this layer in 10–15% of the tubular cross sections examined, the contractile cell layer can only be considered to act as a partial permeability barrier to the penetration of substances into the seminiferous epithelium.

One of the puzzling features of spermatogenesis is how such a cyclic process is regulated in a seemingly constant endocrine environment. When local variations in permeability of the contractile cell layer to electron-opaque tracers were first observed, it was tempting to speculate that these regions might be distributed in some consistent relation to particular segments along the spermatogenic wave and that easier access of gonadotrophins to spermatogonia in certain stages of the cycle of the seminiferous epithelium might stimulate their proliferation or differentiation and thus initiate a cycle which would be self-sustaining until mature sperm were released (Fawcett *et al.*, 1970). This possibility can no longer be seriously entertained for in the lanthanum studies reported here, no evidence could be found that sites of greater permeability were related to any particular stage of the cycle.

Where lanthanum gained access to the base of the germinal epithelium it permeated the interspace between spermatogonia and the surrounding Sertoli cells, just as previously reported for ferritin and peroxidase in the guinea pig (Fawcett *et al.*, 1970). Although one or more intercellular clefts between neighboring Sertoli cells communicate with the interspace surrounding each spermatogonium, the lanthanum penetrated into these only for short distances (0.5–3 μ) before being stopped by focal tight junctions on the Sertoli–Sertoli interface. Despite the fact that the area of membrane contact between Sertoli cells is of very limited extent when seen in transverse section, these occluding junctions are evidently not punctate but are long narrow bands that collectively form a continuous and effective barrier around the spermatogonia preventing deeper penetration of markers into the epithelium from the base.

This finding significantly alters our concept of the organization of the seminiferous tubule. The germinal epithelium is composed of a population of fixed supporting elements, the Sertoli cells, and a mobile population of

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dividing and differentiating germ cells whose topographical relations to the fixed elements are constantly changing. The Sertoli cells are the only elements that extend from the base to the free surface of the epithelium. At their base, they arch individually and collectively over the spermatogonia which also rest upon the basement lamina. By this arrangement and by their specialized junction with each other, the Sertoli cells are now found to isolate the spermatogonia in a separate *basal* compartment of the epithelium (Fig. 18). In a colorful account of the structure of the testis, Elftman (1963) spoke of the Sertoli cells "standing on the basement membrane in patterned array not unlike trees in an orchard." The spermatocytes and early spermatids are arranged in vertical rows around the "trunks" or columns of the Sertoli cells. The deeply excavated lateral aspects of the Sertoli cells conform to the convex contours of the differentiating germ cells crowded between them. The germ cells are partially separated from one another by thin laminar Sertoli cell processes. The vertical rows of differentiating germ cells thus occupy what might be called an *adluminal* compartment around and between the columnar Sertoli cells, while the spermatogonia occupy a *basal* compartment between the Sertoli cells and the basal lamina.

The full functional significance of the isolation of the spermatogonia in a separate compartment has yet to be elucidated. It is apparent, however, that blood-borne nutrients and gonadotrophins which successfully traverse the contractile cell layer, have direct access to the spermatogonia via the surrounding intercellular cleft and through the basal lamina. On the other hand, substances reaching later stages of the germ cell line probably have to traverse the Sertoli cell cytoplasm, owing to the occlusion of the intercellular clefts radiating from the spermatogonia to the adluminal regions of the epithelium.

The observation that the rete testis fluid contains very little protein and only traces of immunoglobulin (Johnson and Setchell, 1968) would lead one to expect that the blood–testis barrier would protect the germinal epithelium against immunological damage. The fact is, however, that the guinea pig and some other species are sensitive to immunization with testis and complete adjuvant (Voisin and Toullet, 1968). Since the initial response seems to be in the rete (Waksman, 1959) where the barrier is relatively ineffective, it may be that some of the damage to the tubules is a secondary effect of distal obstruction. On the other hand, it is quite likely that an early effect of iso-immunization is to alter the cell-to-cell junctions in the adventitial tissue of the tubules and possibly those within the epithelium, thus breaking down the blood–testis barrier (Johnson, 1970). This interpretation derives indirect support from recent observations suggesting that the primary site of action of cadmium is on the boundary tissue of the tubules. Johnson (1969) reported that acriflavine staining of the tubules occurs 24 hr following cadmium treatment and Westmoreland (1970) has found that lanthanum freely penetrates the myoid layer of guinea pig seminiferous tubules a few hours after cadmium administration.

It is obvious that the physiological segregation of the spermatogonia from the remainder of the epithelium cannot be maintained indefinitely and that the pattern of Sertoli cell junctions must be modified at certain stages of the cycle to allow preleptotene spermatocytes to move from the basal to the adluminal compartment. With the displacement of these cells from the basal lamina and their movement toward the lumen it seems likely that processes of neighboring Sertoli cells move in beneath the preleptotene spermatocytes and, coming into contact, reform tight junctional complexes, thus restoring the isolation of the spermatogonia in the basal compartment.

Previous considerations of the extensive

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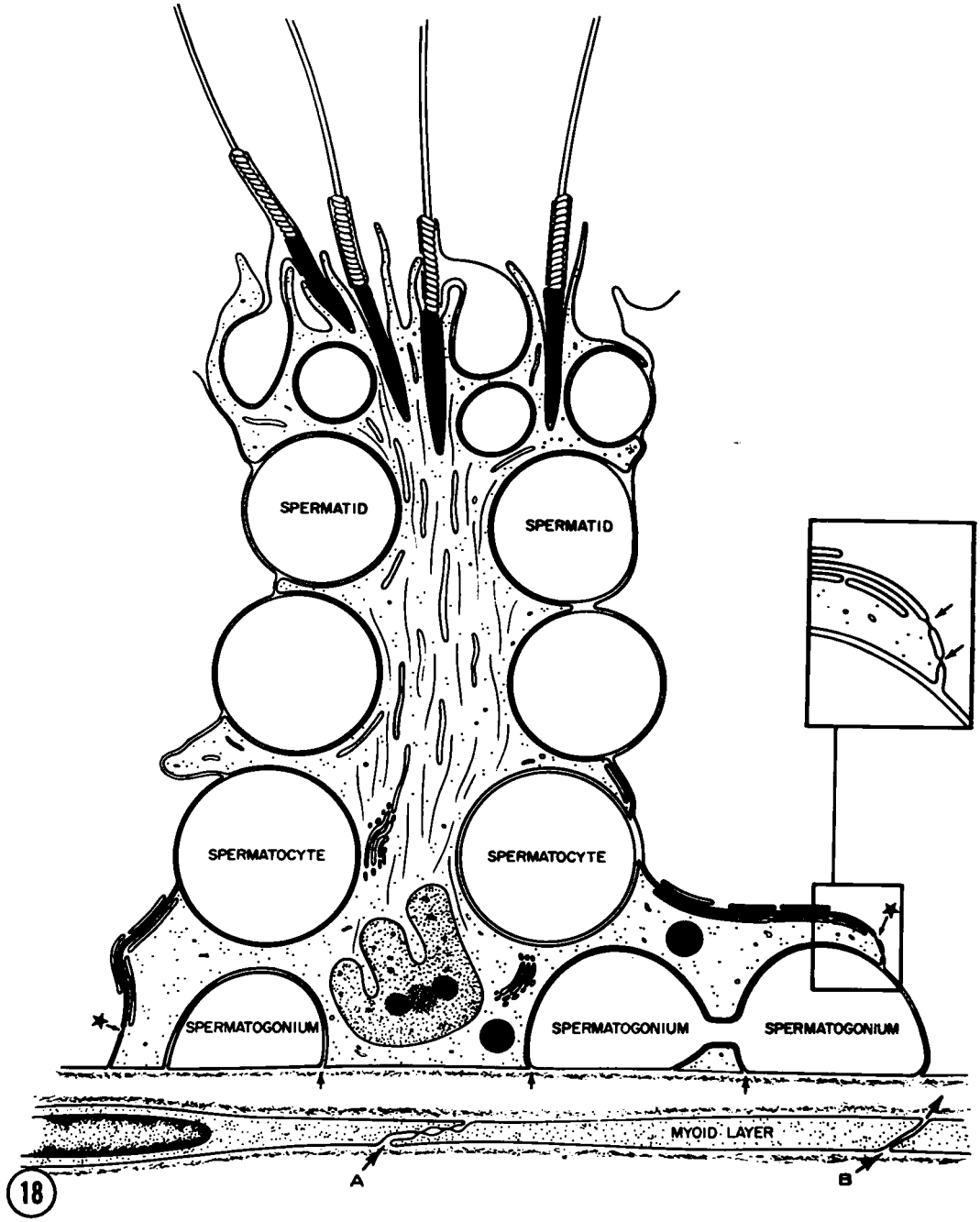

FIG. 18. A diagram depicting the localization of the blood–testis barrier and the compartmentalization of the germinal epithelium by tight junctions between adjacent Sertoli cells. Note the germ cells and their relationship to a columnar Sertoli cell. The primary barrier to substances penetrating from the interstitium is the myoid layer. The majority of cell junctions in this layer are closed by a tight apposition of membranes as indicated at A. Over a small fraction of the tubule surface, the myoid junctions exhibit a 200-Å wide interspace and are therefore open as depicted at B. Material gaining access to the base of the epithelium by passing through open junctions in the myoid layer is free to enter the intercellular gap between the spermatogonia and the Sertoli cells. Deeper penetration is prevented by occluding junctions (stars) on the Sertoli–Sertoli boundaries. These tight junctions constitute a second and more effective component of the blood–testis barrier. In effect, the Sertoli cells and their tight junctions delimit a *basal* compartment in the germinal epithelium, containing the spermatogonia and early preleptotene spermatocytes, and an *adluminal* compartment, containing the spermatocytes and spermatids. Substances traversing open junctions in the myoid cell layer have direct access to cells in the basal compartment, but to reach the cells in the adluminal compartment, substances must pass through the Sertoli cells.

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Sertoli cell junctions which exhibit associated subsurface cisternae favored the interpretation that these might be involved in the cell-to-cell communication necessary for the regional synchronization of the developmental events in spermatogenesis (Flickinger and Fawcett, 1967). Microelectrode experiments have since been carried out to test this hypothesis with the finding that Sertoli cells are electrically coupled over rather long distances irrespective of the boundaries between successive segments of the wave (Spitzer, 1969). Thus the discontinuities between the successive regions of synchronized development in the spermatogenic wave cannot be attributed to segmental regions of Sertoli cell coupling, separated by intervening zones of higher electrical resistance. The results of the present study indicate that the widespread coupling found throughout the epithelium is attributable to the numerous gap junctions and focal tight junctions found on the boundaries between Sertoli cells.

The most distinctive feature of the Sertoli–Sertoli junctions is not the localized tight junctions or the gap junctions but the extensive junctions where the membranes, 90 Å apart, are flanked by bundles of filaments and subsurface cisternae. The significance of this unique junction remains obscure but it does not seem unreasonable to relate it to a unique feature of this epithelium, namely the requirement for continuous upward movement of a population of differentiating germ cells in relation to a population of fixed supporting cells. It is conceivable that when processes from two neighboring Sertoli cells meet below a germ cell, the formation of such junctions and their extension from base toward lumen would *zipper up* the junction and tend to move the cell toward the lumen. This becomes more plausible when one considers that the germ cells are not free to move actively as individuals. The spermatogonia occur in groups of four or more while the spermatocytes and spermatids are found in much larger clusters; furthermore, protoplasmic bridges occur between the cells of the same type (Fawcett *et al.*, 1959). These large irregularly shaped clones of germ cells could only be moved toward the lumen by active changes in the form and interrelations of the surrounding Sertoli cells. The presence of the filaments and the fluid-filled cisternae may endow the ectoplasmic zone of the Sertoli cell with the necessary turgor and contractility to accomplish this work.

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